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Activation of ErbB2 by 2-methyl-1,4-naphthoquinone (menadione) in human keratinocytes: Role of EGFR and protein tyrosine phosphatases

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Abstract **Activation of ErbB receptor tyrosine kinases triggers multiple signaling pathways that regulate cellular proliferation and survival. We here demonstrate that ErbB2 is activated via the epidermal growth factor receptor (EGFR) upon exposure of cultured human keratinocytes to 2-methyl-1,4-naphthoquinone (menadione). Both ErbB2 and EGFR are shown to be regulated by protein tyrosine phosphatases that are inhibited by menadione, giving rise to the hypothesis that phosphatase inhibition by menadione may result in a net activation of EGFR and an enhanced ErbB2 phosphorylation. Isolated PTP-1B, a protein tyrosine phosphatase known to be associated with ErbB receptors, is demonstrated to be inhibited by menadione.**

Keywords: Menadione; ErbB receptors; Epidermal growth factor; Tyrosine phosphatases; Keratinocytes

1. Introduction

ErbB2 is a member of the ErbB family of receptor tyrosine kinases (RTK) that also comprises the epidermal growth factor receptor (EGFR = ErbB1), ErbB3 and ErbB4. An overexpression of ErbB2 is associated with the development of human cancers, such as breast or ovarian cancer [1,2]. Activation of ErbB2 requires heterodimerisation with other ErbB family members as no high-affinity ligand has been described for ErbB2 [3]. Interaction with a ligand-bound dimerisation partner results in tyrosine phosphorylation of the cytosolic domains due to ErbB tyrosine kinase activity, yielding phosphotyrosine residues that serve as docking sites for signaling proteins [3]. ErbB2 is expressed in human skin [4], and it tends to be overexpressed in basal cell carcinoma (BCC) and downregulated in squamous cell carcinoma (SCC) relative to normal epidermis [5].

In addition to ligand-induced activation, the ligand-independent activation of receptor tyrosine kinases, including EGFR and ErbB2, has been described for skin cells exposed to ultraviolet (UV) radiation and was hypothesised to be due to the inactivation of regulating tyrosine phosphatases by UV-derived reactive oxygen species [6,7]. A similar mechanism for the activation of EGFR-dependent signaling in rat liver epithelial cells was proposed for the vitamin K analog, 2-methyl-1,4-naphthoquinone (menadione), which was demonstrated to inhibit a yet unidentified protein tyrosine phosphatase (PTPase) regulating the EGFR [8,9].

The present study investigates activation of ErbB receptors in human keratinocytes exposed to menadione, focusing on the interaction between ErbB2 and EGFR as well as the role of PTPases. Menadione may serve as a model for environmental naphthoquinone derivatives, such as those found in plant extract-based preparations for topical application to skin, including plumbagin and juglone (from walnut extracts) as well as lawsone (from henna leaves).

We demonstrate that exposure of human keratinocytes to menadione leads to phosphorylation and activation of ErbB2, independent of whether keratinocytes are immortalized and tumorigenic or not. ErbB2 phosphorylation is via EGFR, and both EGFR and ErbB2 phosphorylation are regulated by menadione-sensitive PTPase(s) independent from each other.

2. Materials and methods

2.1. Cell culture

Normal human epidermal keratinocytes (NHEK), HaCaT and SCL-1 cells were a kind gift of Dr. Peter Brenneisen, Düsseldorf, and were held in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, Deisenhofen, Germany) supplemented with (final concentrations) 9% (v/v) fetal calf serum (FCS; BioWest, Frickenhausen, Germany), 2 mM Glutamax (Gibco/Invitrogen, Karlsruhe, Germany), and penicillin/streptomycin (100 U/ml and 0.1 mg/ml, respectively; PAA, Cölbe, Germany) at 37 °C in a humidified atmosphere with 5% (v/v) CO_2.

Cells grown to 90–100% confluence in 28 cm^2-dishes were exposed to menadione (Sigma–Aldrich) diluted in serum-free medium for the times indicated. DMSO was taken as vehicle control in the respective appropriate concentration. EGFR kinase inhibitors (AG1478, compound 56, all from Calbiochem, San Diego, CA, USA) were diluted from stock solutions in DMSO. Accordingly, identical volumes of DMSO were used as solvent controls where appropriate.

2.2. Western blotting and immunoprecipitation

Keratinocytes were grown to 90–100% confluence and serum-starved over night before treatment with menadione. After treatment, cells were lysed in RIPA buffer [20 mM Tris–Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% (w/v) Triton X-100, 1 mM Na_3VO_4, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride], followed by protein determination with a detergent-compatible protein assay (Bio-Rad, Munich, Germany). Appropriate volumes of 2× SDS–PAGE sample buffer [125 mM Tris–HCl, 4% (w/v) SDS, 20% (w/v) glycerol, 100 mM DTT, and 0.2% (w/v) bromophenol blue, pH 6.8] were added to protein extracts (usually 20 μg of protein per sample), followed by heat denaturation of samples at 95 °C for 5 min and

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loading onto SDS–polyacrylamide gels of 8% or 10% (w/v) acrylamide, electrophoresis and Western blotting. For detection of phosphorylated and total ErbB2, rabbit polyclonal anti-phospho-ErbB2 antibodies detecting phosphorylation at Tyr-1139 or Tyr-1248 (both from BioSource; Camarillo, CA, USA) and anti-total-ErbB2 (Upstate Biotechnology, Lake Placid, NY, USA, or Cell Signaling Technology, Beverly, MA, USA) antibodies were used. Similarly, polyclonal anti-phospho-EGFR (Tyr1068) (BioSource International) and anti-total-EGFR (Upstate Biotechnology) antibodies were used at the dilutions recommended by the suppliers. Furthermore, rabbit polyclonal anti-phospho-ERK-1/2 and anti-total ERK-1/2 antibodies (both Cell Signaling Technology) were employed. Horseradish peroxidase-coupled secondary antibodies were from Dianova (Hamburg, Germany) or Pierce (Rockford, IL, USA), chemiluminescence detection reagents from Pierce. For immunoprecipitation of EGFR, cells grown to near confluence in 64 cm^2 dishes were lysed in 500 μl IP buffer (30 mM Tris, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 10 mM NaF, 5 mM Na_3VO_4, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride), collected with a cell scraper and centrifuged (10 min, 20000 × *g*, 4 °C). Supernatants were transferred to fresh reaction cups and analysed for protein content with a detergent-compatible protein reagent (Bio-Rad). 700 μg of protein was then incubated with 4 μg of rabbit polyclonal anti-EGFR antibody (Upstate Biotechnology) and 50 μl of a 50%-slurry of protein A-agarose (washed in IP buffer; Upstate Biotechnology) for 14–20 h at 4 °C under end over end rotation. After a brief centrifugation to pellet the agarose beads, they were washed twice with IP buffer and once with Tris–EDTA (25 mM Tris, pH 6.8, 1 mM EDTA), followed by addition of 50 μl of 4× SDS–PAGE sample buffer and an incubation at 95 °C for 5 min. After centrifugation, 20 μl of the respective supernatant was analysed by SDS–PAGE and Western blotting.

2.3. Assay of ErbB2 and EGF receptor dephosphorylation

Dephosphorylation of ErbB2 as well as EGF receptor was analyzed as described before [9,10]. Briefly, HaCaT cells were grown to 80–100% confluency and serum-starved overnight. ErbB2 and EGF receptor tyrosine phosphorylation was stimulated by incubation in the presence of human recombinant EGF (100 ng/ml, R& D Systems, Minneapolis, MN, USA) for 5 min. The cells were washed with PBS and exposed to menadione (100 μM) or DMSO (solvent control) in serum-free DMEM for the indicated times. Following treatment, cells were washed with PBS briefly and fresh serum-free medium containing the EGFR tyrosine kinase inhibitor AG1478 (Calbiochem/Merck, Darmstadt, Germany; 10 μM) was added to prevent any further EGFR-dependent phosphorylation. After 30 s, medium was quickly removed and cells were lysed in 2× SDS–PAGE sample buffer, followed by SDS–PAGE on a gel of 10% (w/v) acrylamide and Western blotting with detection of phosphorylated ErbB2 and EGFR.

2.4. Phosphatase assay

Tyrosine phosphatase activity of the recombinant C-terminal region of human PTP-1B (Biomol, Hamburg, Germany) was measured using *p*-nitrophenyl phosphate (pNPP, Sigma–Aldrich) as substrate. PTP-1B (0.67 μM in 50 mM HEPES, pH 6.8) was preincubated with either DMSO, menadione, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ, Sigma), or 2,3-bis-[2-hydroxyethylsulfanyl]-1,4-naphthoquinone (NSC95397, Sigma) at the given concentrations for 10 min at room temperature in a volume of 50 μl and then added to 750 μl of pNPP/HEPES (2 mM pNPP in 50 mM HEPES buffer, pH 6.8) preequilibrated to 37 °C. The subsequent increase in absorbance at 405 nm (associated with the formation of *p*-nitrophenolate) was monitored, and the formation of *p*-nitrophenol/min calculated using an absorption coefficient of 18000 M^{-1} cm^{-1} after correction for *p*-nitrophenol ionisation.

3. Results and discussion

3.1. Activation of ErbB2 by menadione

Exposure of human keratinocytes to menadione (100 μM) resulted in strong tyrosine phosphorylation of ErbB2, as demonstrated employing antibodies specifically recognizing phosphorylated Tyr-1139 (Fig. 1A) or phosphorylated Tyr-1248 (see below, e.g. Fig. 1C). Similar to ErbB2, tyrosine phosphorylation of EGFR was also elicited in cells exposed to menadione, which is in line with previous reports on EGFR activation by menadione in rat liver epithelial cells [8]. Furthermore, signaling cascades downstream of ErbB2 and EGFR, such as those resulting in activation of extracellular signal-regulated kinase (ERK)-1 and ERK-2, were activated (Fig. 1B). ErbB2 and EGFR phosphorylation signals induced by exposure to menadione were comparable in intensity to those caused by exposure of cells to EGF. Activation of ErbB2 was elicited at concentrations of menadione starting at 10 μM (data not shown) and was detectable after 10 min of exposure (Fig. 1C). When menadione was used at 50 μM rather than 100 μM to yield a slightly protracted cellular response to exposure, it became evident that tyrosine phosphorylation of ErbB2 continuously increased over at least 2 h (Fig. 1C), pointing to either the gradual and slow intracellular accumulation of menadione or to the slow accumulation of an active intermediate generated by metabolism of menadione.

ErbB2 and EGFR activation was similarly strong in primary normal human epidermal keratinocytes (NHEK), in immortalized but non-tumorigenic human keratinocytes (HaCaT) as well as in immortalized and tumorigenic human keratinocytes (squamous cell carcinoma, SCL-1; Fig. 1A).

3.2. Menadione activates ErbB2 via EGFR

Activation of ErbB2 requires heterodimerization with other ErbB family members, including EGFR, which is a known dimerisation partner. In fact, EGF activates ErbB2 in NHEK, HaCaT and SCL-1 cells (Figs. 1A, 2A), which is blocked by inhibitors of the EGFR tyrosine kinase activity, AG1478 and "compound 56" (Fig. 2A) at concentrations preventing activation of EGFR (Fig. 2B). A significant unspecific inhibition of ErbB2 by these inhibitors is unlikely as the employed concentrations (10 μM of both AG1478 or compound 56) were above IC_{50} values for inhibition of EGFR in vitro (AG1478: 3 nM, cpd 56:6 pM) to ensure a most efficient blockade of EGFR, and at the same time well below IC_{50} values for inhibition of ErbB2 (AG1478: >100 μM) [11,12]. Furthermore, inhibitors of ErbB2 tyrosine kinase activity, AG825 (IC_{50}^{ErbB2}: 0.35 μM; IC_{50}^{EGFR}: 19 μM) and AG879 (IC_{50}^{ErbB2}: 1 μM; IC_{50}^{EGFR}: >500 μM) [11], neither blocked menadione-induced nor EGF-induced ErbB2 tyrosine phosphorylation if added to cells in concentrations of 10 μM (data not shown), implying again that a kinase other than ErbB2 itself is responsible for ErbB2 phosphorylation. In line with these data pointing to an interaction of ErbB2 with EGFR, phospho-ErbB2 was coimmunoprecipitated with phospho-EGFR from lysates of EGF-stimulated cells (Fig. 2C, left panel). Surprisingly, ErbB2 was coprecipitated with EGFR even in unstimulated cells (Fig. 2C, right panel, lane 3), implying that preformed ErbB2/EGFR heterodimers exist that are not extensively tyrosine-phosphorylated. It was previously demonstrated that EGFR homodimerisation not necessarily requires ligand binding [13], so the same might also apply for heterodimerization. Interestingly, EGFR/ErbB2 heterodimers, although present, do not appear to be the major form of EGFR occurring in human keratinocytes as EGFR phosphorylation is also not blocked by the above-mentioned ErbB2 tyrosine kinase inhibitors (data not shown).

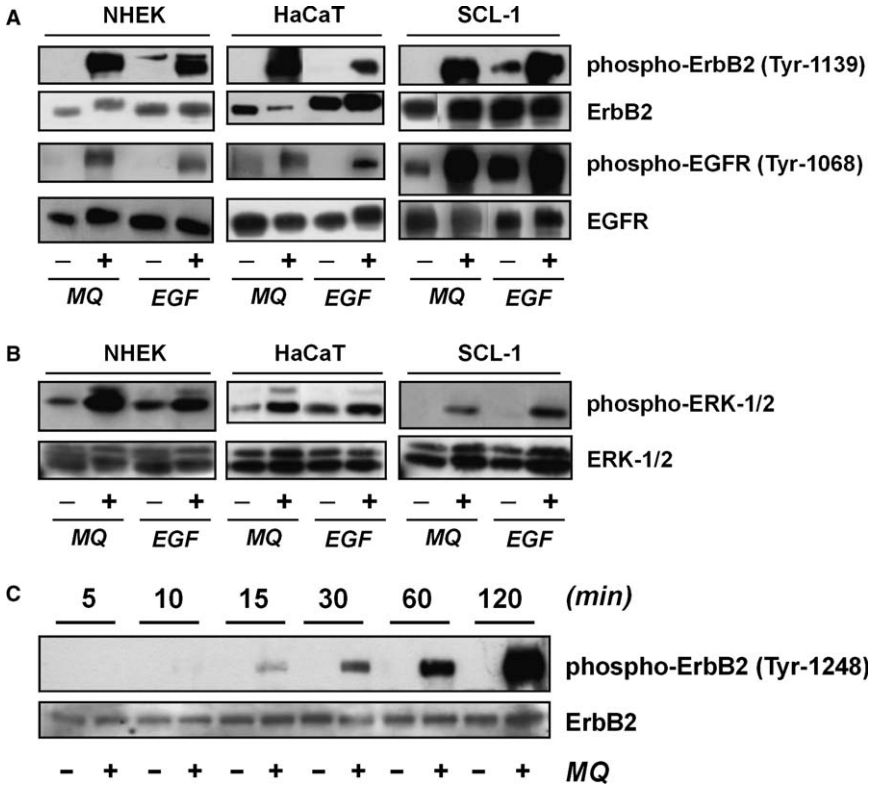

Fig. 1. Activation of ErbB2 and EGFR as well as downstream signaling in human keratinocytes exposed to menadione. Normal human epidermal keratinocytes (NHEK), HaCaT human keratinocytes and human squamous cell carcinoma cells (SCL-1) were serum-starved overnight, followed by exposure to menadione (MQ, 100 μM; DMSO was used as solvent control in these experiments) or EGF (100 ng/ml) in serum-free cell culture medium for 15 min. Analysis of ErbB2 and EGFR phosphorylation (A) as well as of ERK-1 and ERK-2 (B) phosphorylation was done by Western blotting, employing antibodies specifically recognizing the respective phosphorylated protein forms. Gel loading was controlled by analysis of total levels of ErbB2, EGFR or ERK-1/2. (C) Serum-starved HaCaT cells were exposed to 50 μM menadione for the times indicated, lysed and analyzed for ErbB2 phosphorylation by Western blotting. DMSO served as solvent control (–). Data are representative of three independent experiments yielding similar results.

ErbB2 and EGFR activation by menadione in HaCaT cells was partly reversed by postincubation with *N*-acetyl cysteine (NAc, Fig. 3), indicating that the oxidation of an essential cysteine residue to the level of a disulfide or sulfenic acid (sulfenate) is involved in menadione-induced ErbB2/EGFR activation. This oxidative component in ErbB activation by menadione in HaCaT cells may result from redox cycling of the quinone with concomitant generation of reactive oxygen species (ROS). Indeed, the generation of ROS as well as the depletion and oxidation of glutathione in rat hepatocytes exposed to menadione were described already in the early 1980s [14–16]. In line with this, exposure of HaCaT cells to menadione resulted in an enhanced oxidation of glutathione to glutathione disulfide that was detectable already after 15 min of exposure to menadione (data not shown). The steady increase in ErbB2 phosphorylation during longer exposure of the cells to menadione (Fig. 1C) which was postulated above to be due to the continuous formation and accumulation over time of an active agent in the cells would be in accordance with such generation of ROS during menadione redox cycling.

3.3. Tyrosine phosphatase inhibition by 1,4-naphthoquinones

Menadione is capable of inhibiting PTPases in cell-free systems [17] as well as in cell culture [8,18]. In order to test for a modulation by menadione of PTPases that regulate ErbB2 tyrosine phosphorylation, HaCaT cells were stimulated with EGF to induce strong tyrosine phosphorylation of ErbB2 (Fig. 4A, lane 2 vs. lane 1). Addition of the EGFR tyrosine kinase inhibitor AG1478 to block further EGFR-dependent phosphorylation caused a rapid decline in ErbB2 tyrosine phosphorylation (lane 4 vs. 2), pointing to the presence of PTPases that dephosphorylate ErbB2. With menadione, however, ErbB2 remained phosphorylated, even in the presence of AG1478 (lane 6 vs. 4), indicating that PTPases are prevented from dephosphorylating the receptor. As demonstrated in Fig. 4B, dephosphorylation of phospho-EGFR was also impaired by menadione, which is in line with previous data on the inactivation of PTPases regulating EGFR phosphorylation by menadione in rat liver epithelial cells [8]. A time-course analysis of phosphatase activity in cells exposed to menadione (Fig. 4C) revealed that inhibition by menadione of PTPase(s) targeting ErbB2 was detectable after 10, 30 and 60 min of incubation (lane 9 vs. 8). The inhibition elicited by an exposure to menadione (10 min) was not relieved by washing off the quinone and incubating the cells for another 60 min prior to addition of AG1478 and cell lysis (Fig. 4C, bottom). Interestingly, after 30 min of exposure, menadione was capable not only of activating ErbB2 without EGF but to also keep it activated in the presence of AG1478 (lane 11 vs. 10).

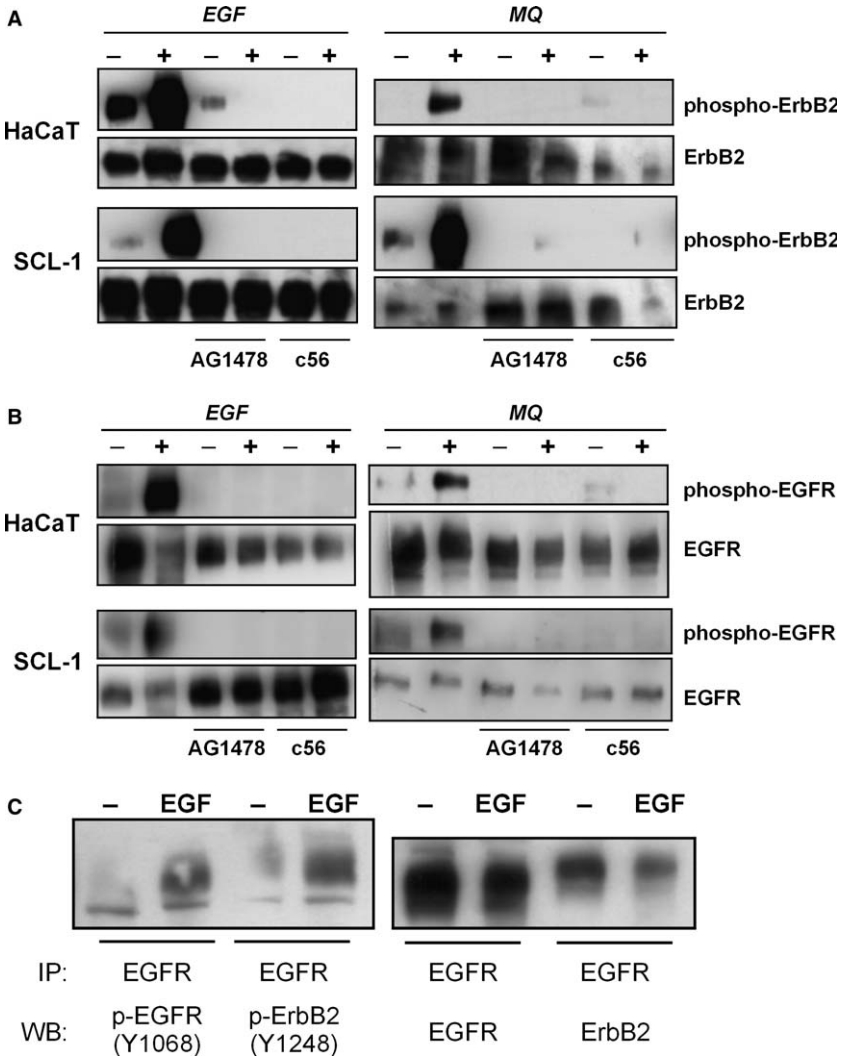

Fig. 2. Role of EGFR in menadione-induced ErbB2 activation. HaCaT and SCL-1 cells were serum-starved overnight and incubated with DMSO (solvent control) or the EGFR inhibitors AG1478 (10 μM) or compound 56 (c56, 10 μM) for 30 min prior to exposure of cells to menadione (MQ, 100 μM; DMSO was used as solvent control) or EGF (100 ng/ml) in serum-free cell culture medium for 15 min in the continued presence of the inhibitors. Analysis of ErbB2 (A) or EGFR activation (B) was done by Western blotting employing appropriate antibodies specifically recognizing the respective receptor phosphorylated at Tyr-1248 (ErbB2) or Tyr-1068 (EGFR). (C) Immunoprecipitation of EGFR in serum-starved HaCaT cells treated with EGF (100 ng/ml; 15 min) where indicated and analysis of EGFR phosphorylation as well as of levels of co-precipitated total as well as phosphorylated ErbB2. Data are representative of at least three independent experiments with similar results.

We have previously postulated that PTPase inhibition by menadione in rat liver epithelial cells is predominantly by arylation [8]. In order to test the hypothesis that PTPase inactivation by menadione and other 1,4-naphthoquinones is mainly by arylation, the inactivation of an isolated PTPase known to associate with and to regulate phosphorylation of EGFR and other receptor tyrosine kinases [19,20], the fragment containing the catalytic domain of recombinant human PTP-1B (residues 1–322) was incubated with menadione and two other 1,4-naphthoquinone derivatives, 2,3-dimethoxy-1,4-naphthoquinone (DMNQ) and NSC95397. The latter was analysed for reasons of comparison as it was described as a most efficient inhibitor of cdc25 as well as other PTPases [21] and to modulate EGFR tyrosine phosphorylation [22]. All three compounds inhibited PTP-1B (Fig. 5), with NSC95397 being the most effective. IC_{50} values under the given conditions (0.67 μM of PTP-1B were incubated with quinones at various concentrations for 10 min) were 0.3, 16.5 and 56.2 μM for

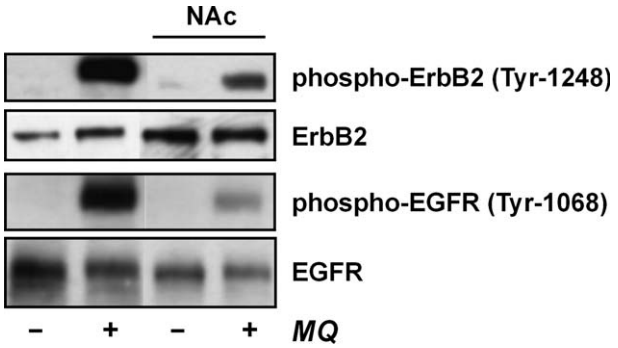

Fig. 3. Attenuation of menadione-induced ErbB2 activation by *N*-acetyl cysteine. HaCaT cells were serum-starved overnight, exposed to menadione (MQ, 100 μM) or DMSO (solvent control) for 15 min, followed by a postincubation with serum-free medium with or without *N*-acetyl cysteine (NAc; 3 mM) for 30 min prior to lysis and Western blot analysis of ErbB2 and EGFR phosphorylation.

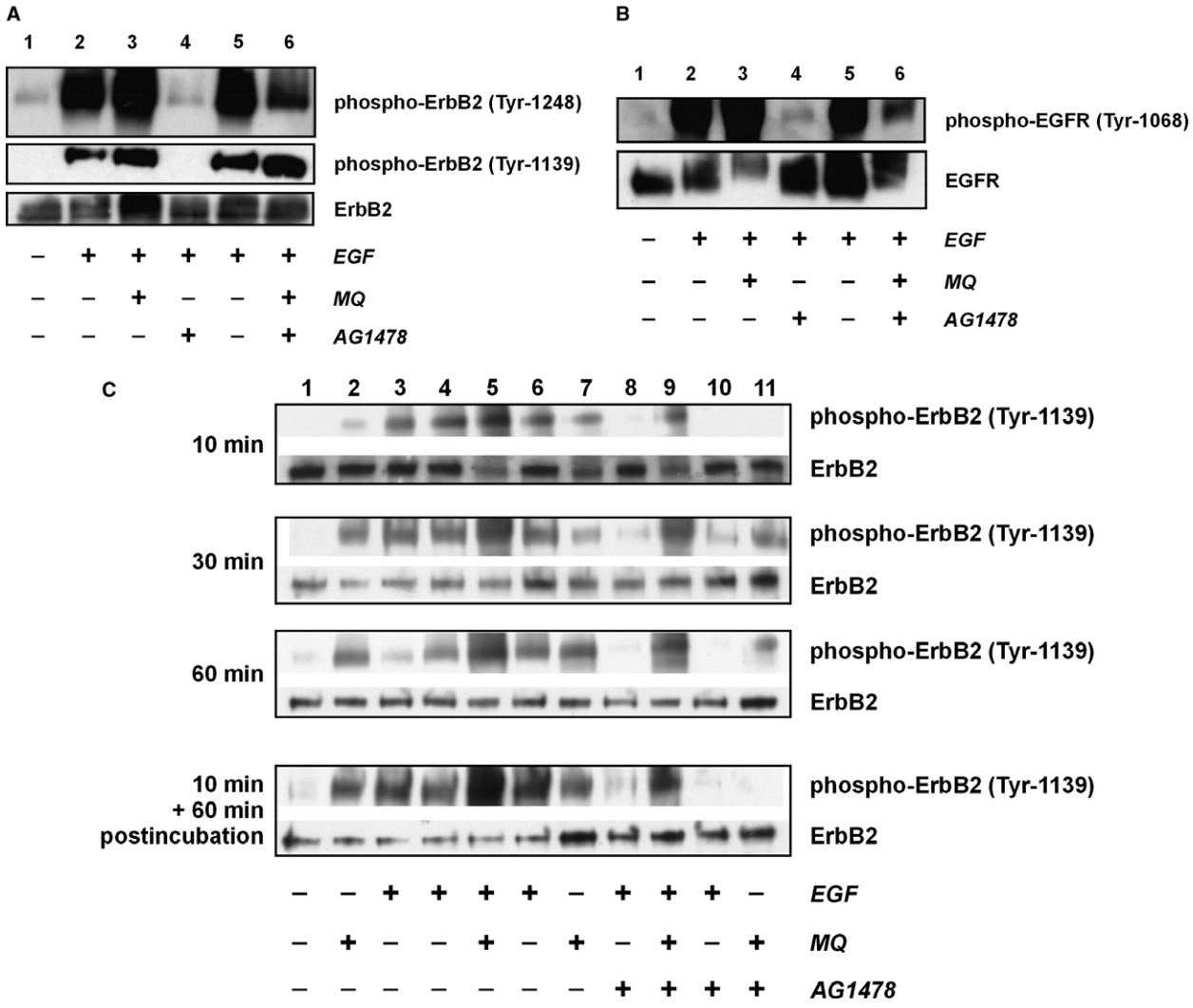

Fig. 4. Inhibition of tyrosine phosphatase regulating ErbB2 by menadione. Serum-starved HaCaT cells were stimulated with EGF (100 ng/ml) for 5 min. Cells were washed with PBS and treated with 100 μM menadione (MQ) for 15 min where indicated. Buffer was removed, and fresh serum-free medium containing the EGFR tyrosine kinase inhibitor AG1478 (10 μM) was added to prevent any further EGFR-dependent phosphorylation. After 30 s, medium was removed quickly, and cells were lysed, followed by Western blotting with detection of ErbB2 phosphorylated at Tyr-1248 or Tyr-1139 (A) as well as of EGF receptor phosphorylated at Tyr-1068 (B). The levels of total ErbB2 and EGFR served as loading controls, respectively. Lane 5: cells were treated with 0.1% DMSO, serving as solvent control for menadione. (C) HaCaT cells were treated as in (A), but exposure to menadione was for 10, 30 or 60 min. Alternatively, a 10 min exposure to MQ was followed by 60 min incubation in menadione-free medium prior to addition of AG1478. In lanes 2 and 7, lanes 3, 4 and 6 as well as lanes 8 and 10, although with identical labeling, cells were treated with different concentrations of DMSO for different times, which served as vehicle control for AG1478 and/or MQ.

NSC95397, menadione and DMNQ, respectively. DMNQ is a non-arylating quinone, inhibiting PTP-1B significantly less efficiently than menadione. Nevertheless, PTP-1B being inhibited by DMNQ implies that arylation is not a strict requirement for PTPase inhibition by 1,4-naphthoquinones. Rather, 1,4-naphthoquinones appear to be capable of inactivating PTPases by noncovalent binding to the enzyme. Like DMNQ, NSC95397 is a non-arylating naphthoquinone; its potency as an inhibitor of cdc25 PTPases was proposed to be due to the strong noncovalent binding in a position close to the active site of the enzyme, with the two quinone oxygens forming hydrogen bonds with two guanidinium moieties of nearby arginine residues [21]. In line with this, the 1,4-naphthoquinone scaffold was found as a structure common to several cdc25 inhibitory compounds [21]. It may be speculated that a similar mode of inhibition occurs with naphthoquinones and PTP-1B.

4. Conclusions

It is demonstrated here that ErbB2 is activated in human keratinocytes exposed to menadione, a 1,4-naphthoquinone derivative; this activation is via EGFR which interacts with ErbB2. Menadione inhibits PTPases in cell-free systems and in cultured cells, suggesting that ErbB activation by menadione occurs indirectly by inhibition of PTPases negatively regulating both EGFR and ErbB2. These findings imply that other 1,4-naphthoquinones that may get in contact with human skin could cause the same ligand-independent activation of ErbB receptors that is found with menadione. Because of the prominent role of ErbB receptors in the regulation of proliferation and skin carcinogenesis this may raise concerns with regard to a possible skin cancer promoting effect of topically applied 1,4-naphthoquinone derivatives, occurring, for example, in

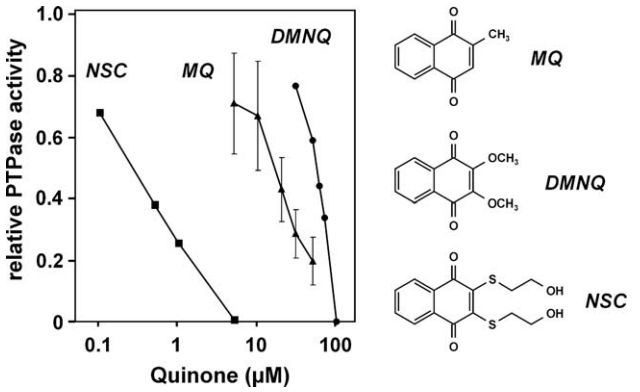

Fig. 5. Inhibition of PTP-1B by naphthoquinones. Recombinant fragment containing the catalytic domain of human PTP-1B was incubated in the presence of menadione (MQ), 2,3-dimethoxy-1,4-naphthoquinone (DMNQ), or 2,3-bis-[2-hydroxyethylsulfanyl]-1,4-naphthoquinone (NSC) prior to phosphatase activity determination. Data are means of at least 3 independent measurements ± S.E.M. (MQ) or means of two independent measurements (DMNQ, NSC). A relative PTPase activity of 1.0 corresponds to a specific activity of 14.6 ± 5.0 μmol/min per mg protein (means ± S.D., $n = 19$) with *p*-nitrophenyl phosphate as a substrate.

henna preparations. Two possible interventional approaches are introduced in the present study; as ErbB2 activation by menadione is via EGFR, the inhibition of EGFR will block activity of EGFR as well as activation of ErbB2 (Fig. 2); several ErbB inhibitors exist and are being developed for use in humans [2]. Secondly, EGFR and ErbB2 activation by menadione may be attenuated with *N*-acetyl cysteine (Fig. 3), suggesting that antioxidative strategies could be successful in blunting 1,4-naphthoquinone-induced ErbB activation in human skin.

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